Simultaneous Tether Extraction Contributes to Neutrophil Rolling Stabilization: A Model Study

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ABSTRACT Neutrophil rolling is the initial step of neutrophil recruitment to sites of inflammation. During the rolling, membrane tethers are very likely extracted from both the neutrophil and the endothelial cell lining of vessel walls. Here, we present a two-dimensional neutrophil-rolling model to investigate whether and how membrane tethers contribute to stable neutrophil rolling. In our model, neutrophils are assumed to be rigid spheres covered with randomly distributed deformable microvilli, and endothelial cells are modeled as flat membrane surfaces decorated with evenly distributed ligands. The instantaneous rolling velocity and other unknowns of the model are calculated by coupling the hydrodynamic resistance functions, the geometric relationships, and the constitutive equations that govern microvillus extension and tether extraction. Our results show that glutaraldehyde-fixed neutrophils (without microvillus extension or tether extraction) roll unstably on a P-selectin-coated substrate with large variance in rolling velocity. In contrast, normal neutrophils roll much more stably, with small variance in rolling velocity. Compared with tether extraction from the neutrophil alone, simultaneous tether extraction from the neutrophil and endothelial cell greatly increases the lifetime of the adhesive bond that mediates the rolling, allows more transient tethers to make the transition into stable rolling, and enables rolling neutrophils to be more shear-resistant.

INTRODUCTION

Neutrophil recruitment to sites of inflammation involves four sequential steps: attachment, rolling, firm adhesion, and diapedesis. The rolling of neutrophils in the blood vessel is an essential step mediated mainly by selectins, namely L-, E-, and P-selectins, and their ligands (1,2). The interaction between selectins and their ligands is governed kinetically by large on and off rates, which can be greatly affected by the hydrodynamic forces exerted by the blood flow and mechanical properties of the cells and molecules involved. In other words, rolling is a dynamic process controlled by biological, chemical, and mechanical factors acting in concert.

The rolling of neutrophils was first reproduced in vitro in a flow-chamber study wherein a leukocyte suspension flowed over a selectin-coated substrate (3). Subsequently, the rolling process was described with a mathematical model that incorporated hydrodynamics and biochemical kinetics (4). Since then, many experimental and analytical studies have contributed to an improved understanding of neutrophil rolling on the endothelium in the blood stream. At high vessel flow rates with attendant high wall shear stresses, fast rolling and large variations in the rolling velocity are expected, because the lifetime of a selectin bond decreases exponentially in response to increasing pulling forces exerted on the connecting tether (5). However, the increase in the rolling velocity as a function of increasing vessel flow rate is much more prominent as observed in a cell-free rolling system, where rigid receptor-coated microspheres roll on ligand-coated substrates (6,7). When normal neutrophils roll on ligand-coated substrates, the rolling velocity varies only in a small range over a broad range of wall shear stress (7–9). Therefore, these studies indicate that, in addition to molecular properties, cellular properties including tether extraction and cytoskeletal deformation are equally important in mediating the rolling process.

Using the micropipette aspiration technique (MAT), Shao et al. first demonstrated that neutrophil microvilli stretched like a spring at small pulling forces, whereas membrane tethers (cylindrical membrane tubes tens of nanometers in diameter) were extracted at large pulling forces (10,11). If double tethers (two tethers in parallel from one cell) were extracted from neutrophils, these two tethers would act as though they were independent of each other mechanically (12). Therefore, it was suggested that membrane tethers were very likely extracted from neutrophils during their rolling, where blood flow could exert large pulling forces on them. In a later study, using differential interference contrast microscopy, membrane tethers were indeed visualized when neutrophils rolled on activated platelets in a flow chamber (13). In another study carried out using the same technique, Park et al. showed distinct rolling patterns among microspheres, fixed neutrophils, and normal neutrophils during their rolling on selectin-coated substrates (9). More recently, Ramachandran et al. showed that membrane tether structures were sensitive to changes in shear stress and that, at high shear stress, the number of tethers pulled from neutrophils was greater and the tethers had developed more complex tether structures to stabilize rolling (14). Based on these findings, three new theoretical models that take account of

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neutrophil microvillus deformation and tether extraction have emerged (15–17).

When a neutrophil rolls on the endothelium, the force that causes tether extraction from the neutrophil is also exerted on the endothelium. Using the MAT, Girdhar and Shao showed that membrane tethers could also be extracted from human umbilical vein endothelial cells with pulling forces comparable to those extracting tethers from neutrophils (18). Therefore, during the rolling process, simultaneous tether extraction from both the neutrophil and endothelium (two tethers in series, one from each cell) is highly likely. Simultaneous tether extraction can decrease the pulling force exerted on the adhesive bond much faster and thus can further stabilize the rolling process compared with tether extraction from the neutrophil alone. To our knowledge, no modeling study of simultaneous tether extraction has been attempted.

In this article, we first simulate the rolling of glutaraldehyde-fixed and normal neutrophils on ligand-coated substrates and compare the two cases to investigate how tether extraction from the neutrophil influences the rolling process. We then simulate the rolling of normal neutrophils on some endothelial cells to investigate whether and how simultaneous tether extraction can further stabilize the rolling and help arrest neutrophils on the endothelium. Our simulation results clearly indicate that simultaneous tether extraction helps stabilize the rolling process by increasing the lifetime of the adhesive bond that mediates this process.

MODEL DESCRIPTION

The model described below is a modified version of the one developed by Hammer et al. (4,15,19–21). The model geometry is shown in Fig. 1, where a rectangular coordinate system (x,y,z) is defined. We assume that the cell motion is constrained to the xz plane. The spherical neutrophil (radius R) is modeled as a rigid body with deformable protruded microvilli, and the endothelial cell is modeled as having a rigid cytoskeleton and uniformly distributed ligands on its tether-capable membrane surface. We assume that 26 (N_{mv}) microvilli are distributed randomly along the neutrophil perimeter (a circle in the xz plane), and that these microvilli are 0.35 μm long (11,22). Only one type of receptor-ligand bond is included in our model: P-selectin/PSGL-1. The P-selectin molecule consists of an N-terminus, a lectin domain, an epidermal-growth-factor-like (EGF) domain, several consensus repeat units, a transmembrane domain, and an intracellular cytoplasmic tail. The molecular lengths of P-selectin and PSGL-1 are ~40 nm and 60 nm, respectively (23,24). P-selectin is distributed on activated endothelial cells and platelets, whereas PSGL-1 is mainly located on the neutrophil microvilli (25,26). This special arrangement of PSGL-1 facilitates the initial attachment of neutrophils to the endothelium. In our simulation, Poisson sampling is used to distribute PSGL-1 on the microvillus tip with an average of eight receptors per microvillus tip (represented by $\langle r \rangle$).

The neutrophil microvillus is assumed to behave like a soft string, which implies that it cannot resist compression but can resist stretching. Once an attached microvillus is stretched, it is likely that not many new bonds can form on this microvillus because all free receptors will likely move away from the substrate. Because of the short contact time between the microvillus and endothelial cell (on the order of milliseconds), no lateral movement of receptors on the microvillus tip is considered. The minimum gap width that may exist between the neutrophil and endothelial cell is assumed to be 30 nm. Therefore, once the distance between the neutrophil surface and endothelial cell surface reaches this value, it rolls at the same height of 30 nm above the endothelial cell. The Bell model is used to relate the dissociation rate constant (k_r) to the force on the bond (5),

$$k_r = k_r^0 \exp(\gamma_c F / k_B T), \tag{1}$$

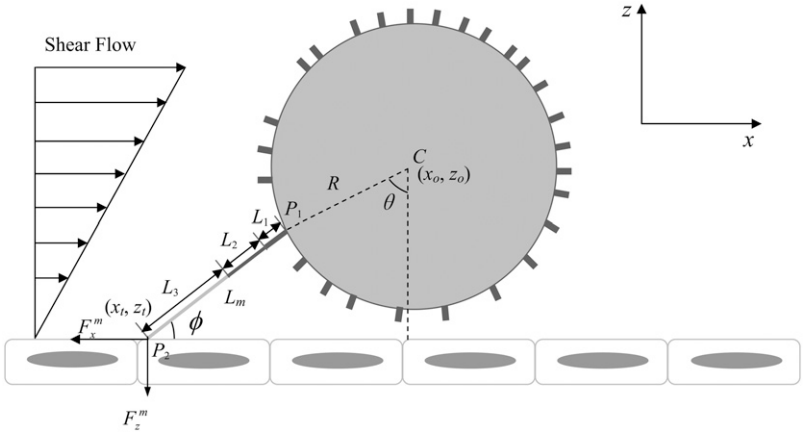

FIGURE 1 Schematic representation of the model geometry (not drawn to scale). Only one pair of simultaneous tethers (one from the neutrophil and the other from the endothelial cell) are shown, although multiple pairs can be present in the simulation. The y axis, which is not shown, forms a right-handed coordinate system with x and z. R is the cell radius. L_1 is the length of the extended microvillus, L_2 is the length of the tether extracted from the neutrophil microvillus, and L_3 is the length of the tether extracted from the endothelial cell. L_m, which is equal to $L_1 + L_2 + L_3$, is the total length of the extended microvillus and extracted tethers. C represents the center of the neutrophil, P_1 is the junction point between the microvillus and neutrophil body, and P_2 represents the attachment point of the tether from the endothelial cell. (x_o, z_o) are the coordinates of C, and (x_t, z_t) are the coordinates of point P_2. θ is the angle between line CP_1 and the negative z direction, and ϕ represents the contact angle between the tether and the endothelial surface. F_x^m and F_z^m are the two components of the pulling force exerted by the blood flow on the composite tether. The force and torque resultants due to the fluid flow are not shown.

where k_r^0 is the reverse rate constant of P-selectin/PSGL-1 binding under zero force, k_B is the Boltzmann constant, T is the absolute temperature, F is the force exerted on the bond, and γ_c is the reactive compliance. It should be noted that this equation is only applicable when the pulling force is constant. However, the pulling force on the P-selectin bond is variable in our simulation. Therefore, Eq. 1 is applied separately in each small time step, where the pulling force does not change much. With a simple Monte Carlo simulation of a bond that obeys Eq. 1, we found that this approach can accurately simulate the behavior of such a bond pulled by an increasing force at a constant loading rate (data not shown).

At the start of a simulation, the neutrophil is positioned above the substrate at a gap of 450 nm (h_{gap}, the length of the microvillus plus the lengths of the P-selectin and PSGL-1). The neutrophil and fluid are assumed to have the same density, so no buoyancy needs to be considered. Before adhesion, the neutrophil translates and rotates freely in the flow. When a receptor on the microvillus and a ligand on the endothelial cell come close enough to bind to each other during a certain time step (Δt), the probability of forming a new bond, P_b, is governed by

$$P_b = 1 - \exp(-k_f \Delta t), \tag{2}$$

where k_f is the association rate of P-selectin/PSGL-1 interaction, which is determined from k_f^0 and [P-selectin], the number of P-selectin per micron squared. Similarly, we can calculate the bond dissociation probability, P_r, at each time step by

$$P_r = 1 - \exp(-k_r \Delta t). \tag{3}$$

If more than one bond is present on a microvillus, we assume all the bonds share the force applied on this microvillus equally.

The hydrodynamic force and torque on the neutrophil can be calculated as those on a rigid microsphere by using hydrodynamic resistance functions (h_i) derived by Zhao et al. (27). These functions were extended from the ones derived by Goldman et al. for a sphere in a shear flow above a substrate (28). In general, the pulling force on a microvillus can be resolved into its normal and tangential components, denoted as F_x^m and F_z^m. The mechanical equilibrium equations of the forces and torques exerted on the neutrophil are

$$h_1 v_x + h_2 \omega_y + h_6 \gamma + \sum_{i=1}^{N} F_i^m \cos\phi_i = 0; \tag{4}$$

$$h_5 v_z + \sum_{i=1}^{N} F_i^m \sin\phi_i = 0; \tag{5}$$

and

$$h_3 v_x + h_4 \omega_y + h_7 \gamma + \sum_{i=1}^{N} [z_o F_i^m \cos\phi_i - (x_o - x_{ti}) F_i^m \sin\phi_i] = 0, \tag{6}$$

where v_x and v_z are the horizontal and vertical velocity components, respectively, of the neutrophil center of mass, ω_y is the rotational velocity of the cell, h_j ($j = 1, 2\ldots7$) are the hydrodynamic resistance functions, ϕ_i is the contact angle for the ith attached microvillus, as shown in Fig. 1 ($i = 1, 2\ldots N$), x_o and z_o are the coordinates of the cell center, x_{ti} is the x coordinate of the tip of the ith attached microvillus, γ represents the wall shear rate, and N is the total number of stretched microvilli.

The kinematic relationships of the model require that

$$v_x - v_{mi}\cos\phi_i + \omega_{pyi} L_{mi} \sin\phi_i - R\omega_y \cos\theta_i = 0, \tag{7}$$

and

$$v_z - v_{mi}\sin\phi_i - \omega_{pyi} L_{mi} \cos\phi_i + R\omega_y \sin\theta_i = 0, \tag{8}$$

where

$$v_{mi} = \frac{dL_{mi}}{dt}, \tag{9}$$

and

$$\omega_{pyi} = \frac{d\phi_i}{dt}. \tag{10}$$

L_{mi} is the total length of the extended microvillus and extracted tethers of the ith attached microvillus, so the total number of kinematic equations is $2N$.

The constitutive equations for the ith microvillus extension and tether extraction from the neutrophil and endothelial cell are as follows:

1), neutrophil microvillus extension,

$$F_i^m = k_1 (L_1^i - L_0)\ (i = 1, 2\ldots N); \tag{11}$$

2), neutrophil tether,

$$F_i^m = F_{01} + 2\pi\mu_1 \frac{dL_2^i}{dt}\ (i = 1, 2\ldots N_n); \tag{12}$$

and

3), endothelial cell tether,

$$F_i^m = F_{02} + 2\pi\mu_2 \frac{dL_3^i}{dt}\ (i = 1, 2\ldots N_e), \tag{13}$$

where F_{01} and μ_1 are the threshold force and effective viscosity for tether extraction from the neutrophil, respectively, F_{02} and μ_2 are the threshold force and effective viscosity for tether extraction from the endothelial cell, respectively, L_1^i represents the ith extended microvillus length, L_0 is the initial length of the microvillus (the same for all microvilli), L_2^i represents the ith tether length from the neutrophil, L_3^i is the ith tether length from the endothelial cell, N_n represents the number of tethers from the neutrophil, and N_e represents the number of tethers from the endothelial cell. Therefore, the total number of constitutive equations is the summation of N, N_n, and N_e. All the variables in the model, such as the rolling velocity, the tether length, and the forces

exerted on the attached microvilli, can be calculated directly at each time step (10^{-4} s) by solving Eqs. 4–13 simultaneously with the Euler method. When simulating fixed neutrophils rolling on rigid substrates as described by Park et al. (9), we block the possibility of tether extraction from neutrophils, assume an effective hydrodynamic radius for the cell body ($R_{\text{eff}} = R + L_{\text{mv}}$) in our hydrodynamic calculations (4) and increase the extensional stiffness of the microvillus to a very large value (5×10^3 pN/μm). The rigid substrate was simulated by not allowing tether extraction from endothelial cells where P-selectin was uniformly distributed.

In our simulation, the Reynolds number (Re) is defined as $\rho UL/\mu$, where ρ is the fluid density, U is the initial free motion velocity of the cell (approximately equal to the average fluid velocity in the range of $z = h_{\text{gap}}$ and $z = 2R + h_{\text{gap}}$ or the fluid velocity at $z = R + h_{\text{gap}}$), L is the diameter of the neutrophil ($2R$), μ is the dynamic viscosity of the fluid. In most time steps of the simulation, the inertial effects of the fluid and cell body can be ignored because Re is small when the neutrophil is rolling on the endothelium. However, the mechanical equilibrium of the cell body is disturbed whenever an attached microvillus or extracted tether breaks free from the substrate. In that event, the inertial effect of the cell body is considered by using a smaller time step (10^{-6} s) and a group of ordinary differential equations is solved until mechanical equilibrium is restored. The computation was programmed in Matlab and carried out on a Windows PC (Pentium IV 2.8 GHz). We tested our program with smaller time steps, which did not alter the results appreciably. The computation took ~20–30 min to reach $t_{\text{m}} = t_{\text{i}} + 4$ s (t_{i} is the initial time of attachment and t_{m} is the computational time) for cases where $s = 300\ \text{s}^{-1}$ and [P-selectin] = 12 molecules/μm^2. All other parameters used in the computation are listed in Table 1.

RESULTS

Contribution of tether extraction from the neutrophil

For the rolling of normal neutrophils on a rigid substrate as in a flow chamber, Fig. 2 shows the results of a typical case where the site density of P-selectin is 100 molecules/μm^2 and the wall shear rate is 100 s^{-1}. It is seen that once the first bond was formed at ~0.13 s, the velocity of the cell quickly decreased from its free translational velocity of ~317 μm/s to ~2.06 μm/s (Fig. 2 *a*; see also Fig. 3 *b*, which is the corresponding velocity plot). The transition shown here is consistent with what has been observed in flow-chamber experiments (13). Once a cell is adherent to the substrate, the cell is pulled closer to the substrate. Then other microvilli may also adhere to the substrate and some of them are in the front half of the cell body, so all microvilli that are adherent are not initially stretched. As shown in Fig. 2, *b* and *c*, during the stable rolling process, many bonds are formed and as many as eight microvilli are adherent. However, some of these new bonds had no significant effects on the rolling velocity, probably because the number of stretched microvilli is always around three after the rolling is stabilized, as shown in Fig. 2 *c*. Starting at ~3.2 s, the rolling velocity had a few jumps (Fig. 3 *b*) while the total number of bonds increased (Fig. 2 *b*). This is because most of the new bonds formed around this moment were located in the front half of the cell body. Consequently, these bonds had no contribution to the rolling velocity because the microvilli where these new bonds were located were not stretched yet. The jumps were generated by the rupture of adhesion on the stretched microvilli, as shown in Fig. 2 *c*. Once more microvilli were stretched, the rolling velocity quickly decreased again (Figs. 2 *c* and 3 *b*).

TABLE 1 Parameters of the model

Symbol	Definition	Value
R	Neutrophil radius	4.25 μm
γ	Shear rate	50–1000/s
η	Fluid viscosity	0.0011 pN·s/μm^2
N_{mv}	Number of microvilli along the cell perimeter	26
$\langle r \rangle$	Average number of receptors on each microvillus tip	8
L_0	Natural length of the microvillus	0.35 μm
$L_{\text{PSGL-1}}$	Molecular length of PSGL-1	0.06 μm
$L_{\text{P-selectin}}$	Molecular length of P-selectin	0.04 μm
k_{r}^0	Two-dimensional reverse rate constant under zero force	1.0/s
k_{f}^0	Two-dimensional forward rate constant under zero force	0.04 μm^2/s
[P-selectin]	Site density of P-selectin on the substrate	12 or 100* molecules/μm^2
γ_{c}	Reactive compliance of the PSGL-1/P-selectin bond	0.04 nm
k_1	Microvillus stiffness	43 or 5×10^3* pN/μm
μ_1	Effective viscosity of tether extraction from neutrophils	1.75 pN·s/μm
μ_2	Effective viscosity of tether extraction from endothelial cells	0.5 pN·s/μm
F_{01}	Threshold force of tether extraction from neutrophils	45 pN
F_{02}	Threshold force of tether extraction from endothelial cells	50 pN

*When two values are listed, the asterisk marks the value that was used in the simulation of fixed neutrophils.

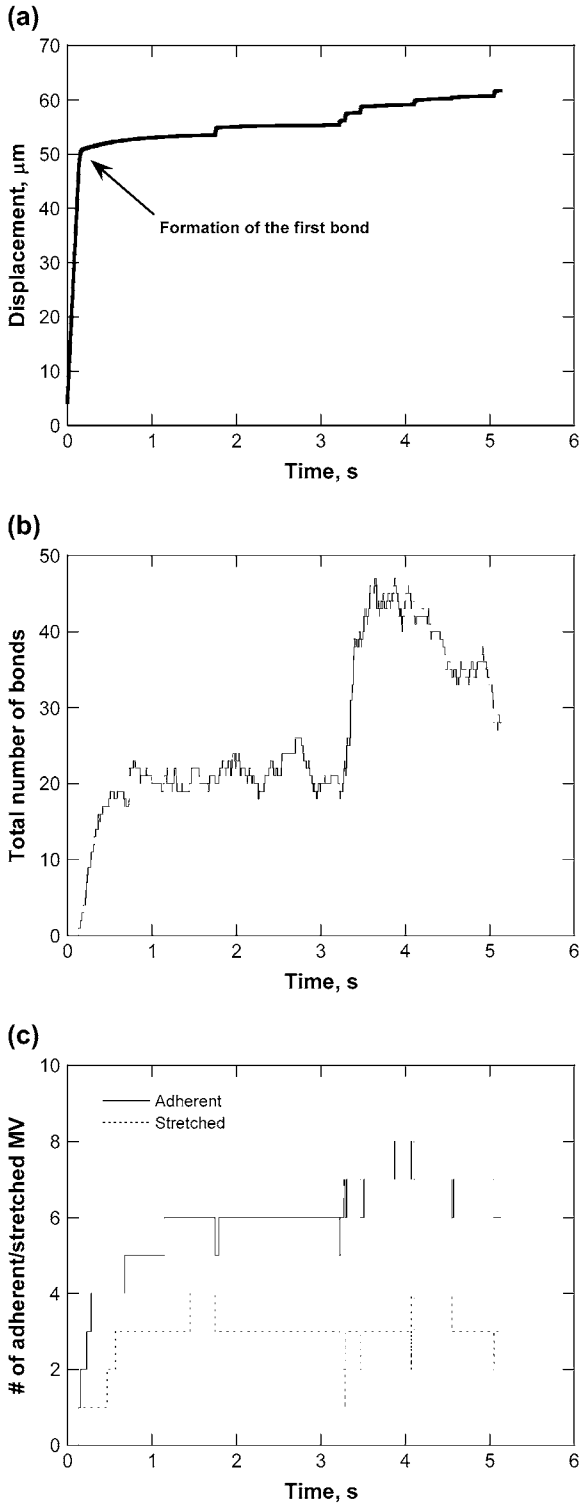

FIGURE 2 The displacement of the neutrophil parallel to the x axis (a), the total number of bonds formed on the cell (b), and the total number of adherent or stretched microvilli (c) over time during the attachment and rolling of a normal neutrophil on a P-selectin-coated substrate. The instantaneous rolling velocity of this cell is shown in Fig. 3 b as a comparison to the rolling velocity of a fixed cell. In c, the solid line represents the number of adherent microvilli and the dotted line represents the number of stretched microvilli. *MV*, microvilli.

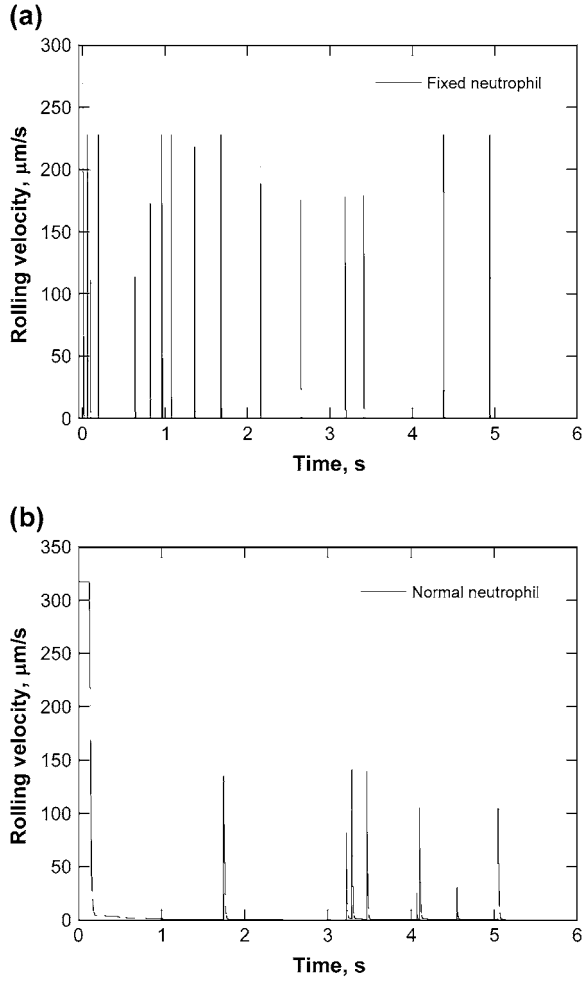

FIGURE 3 The instantaneous rolling velocities of a fixed neutrophil (a) and a normal one (b), respectively, on a rigid substrate where [P-selectin] = 100 molecules/μm^2 and $\gamma = 100$ s^{-1}.

In glutaraldehyde-fixed neutrophils, glutaraldehyde cross-links the cytoskeleton and proteins to alter the mechanical properties of the cells, making them more rigid (9). Hence, fixed neutrophils cannot extend microvilli or protrude tethers when they roll on selectin-coated substrates. As observed in flow-chamber experiments (7,9,29,30), our simulation showed that fixed neutrophils rolled unstably with high variance in rolling velocity. In contrast, normal cells with tether extraction capability rolled very stably with low variance in rolling velocity. Fig. 3, a and b, shows the instantaneous rolling velocities of a fixed neutrophil and a normal one, respectively. Fig. 3 b corresponds to the case shown in Fig. 2. In contrast to the fixed cell, the normal neutrophil has much longer pauses and fewer velocity jumps. These different cell motion patterns are in good agreement with what has been observed in flow-chamber experiments (9). However, the average rolling velocities of these two types of rolling motion do not significantly differ at the two shear rates simulated (Fig. 4 a) ($p > 0.05$ at both shear rates, Student's t-test).

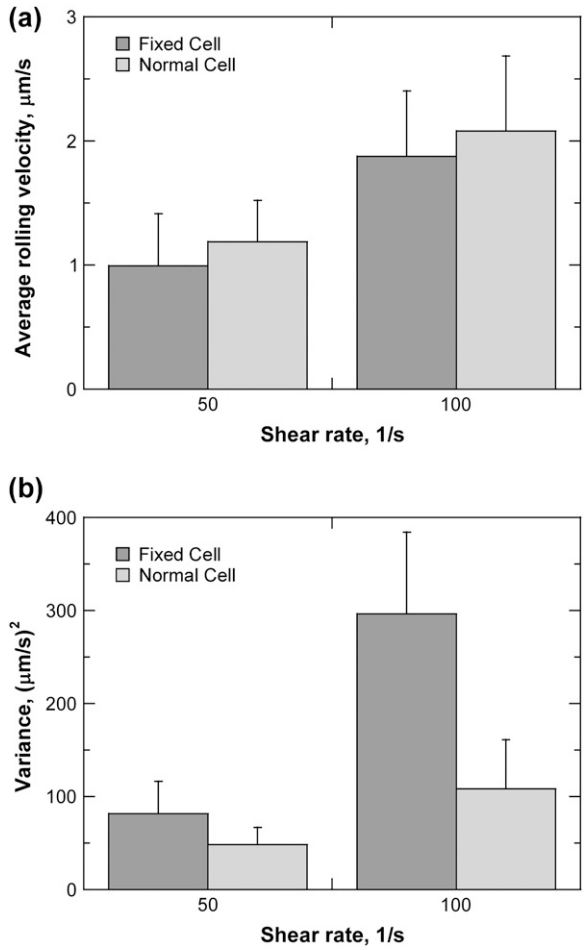

FIGURE 4 The comparison between the mean (*a*) and variance (*b*) of the rolling velocity of the fixed neutrophils and normal ones over the whole rolling duration. The error bars here and in all the following figures represent the standard deviations. At 50 s^{-1}, 30 fixed cells and 30 normal cells were studied; at 100 s^{-1}, 29 fixed cells and 33 normal cells were studied.

This is probably because, once a bond is formed, fixed neutrophils cannot move, due to the lack of tether extraction, and the time duration when they have large rolling velocities is very short. Nevertheless, the variances of the rolling velocities of fixed and normal neutrophils do show a significant difference (Fig. 4 *b*) ($p < 0.01$ at both shear rates, Student's *t*-test). In the case of fixed neutrophils, adhesive bonds were broken often. The fixed cells can roll only because many bonds could form quickly one after another. Therefore, tether extraction is crucial for the stabilization of neutrophil rolling by decreasing the force exerted on the adhesive bond.

Contribution of simultaneous tethers from the neutrophil and endothelial cell

As predicted by Girdhar and Shao (18), membrane tethers can also be extracted from the endothelial cells during the rolling process. To investigate the effect of simultaneous tether extraction on neutrophil rolling, we simulated the rolling of normal neutrophils on the endothelium by incorporating tether extraction from both cell types. In the simulation, if a cell rolls for <1 s and goes back to free motion, we categorize this as a transient tether case; if a cell keeps rolling for >1 s, we categorize the motion as stable rolling; if a cell translates freely in the flow without adhesion for 3 s, we categorize this as free motion. Consequently, all the results can be divided into these three categories: free motion, transient tether formation, and stable rolling. We simulated 100 cells at five different wall shear rates for each type of cell rolling on P-selectin with a site density of 12 molecules/μm^2, which is comparable to the value used in the flow-chamber experiments (9). At shear rates of 100, 200, 300, 400, and 500 s^{-1}, the percentages of cells that rolled stably with simultaneous tether extraction were 72%, 55%, 36%, 26%, and 6%, respectively, whereas the percentages of cells that rolled stably with tether extraction from the neutrophil alone were 66%, 43%, 26%, 8%, and 1%, respectively.

Fig. 5, *a* and *c*, shows the force history of the first adhesive bond of all the simulated adherent cells (transient or stable rolling) at shear rates of 100 and 300 s^{-1} for the case of simultaneous tether extraction. In contrast, Fig. 5, *b* and *d*, shows the force history at the same shear rates for the case of tether extraction from the neutrophil alone. In both cases, at 300 s^{-1}, the force increased from zero to ~200 pN within 0.1 s, then decreased due to microvillus extension and tether extraction. Afterwards, the force declined quasiexponentially toward a plateau, as predicted for single adherent microvilli (11,16,18). In the cases at 100 s^{-1}, the maximum force reached before the decline was only ~80 pN. The force decrease should depend on the total number of stretched microvilli and the type of tether extraction. It is obvious that, in the case of simultaneous tether extraction at 300 s^{-1}, the force on the first adhesive bond decreased much faster (Fig. 5, *a* and *b*) and, on average, the initial bond also lasted longer (see Fig. 7). This stabilizing effect is not as prominent at 100 s^{-1}, which is expected since the pulling force is closer to the threshold force of tether extraction and the tether growth becomes very slow. The complexity of the force history profile shown in Fig. 5 is due to the fact that, for any cell to roll stably, there have to be multiple attached microvilli. The adhesive bonds on some initial tethers do not dissociate until several additional tethers are extracted, and this will help decrease the force load on the initial tether further. On the other hand, the adhesive bonds on some tethers extracted after the initial one could be broken even before the ones on the initial adherent microvillus are. This will in turn increase the force on the initial tether. The magnitude of this increase depends on the shear rate and the broken tether location. Therefore, it is not surprising that a sawtooth pattern was actually obtained in the latter half of the bond lifetime shown Fig. 5. This pattern was not seen in another study, where only one microvillus was adherent during the rolling, although

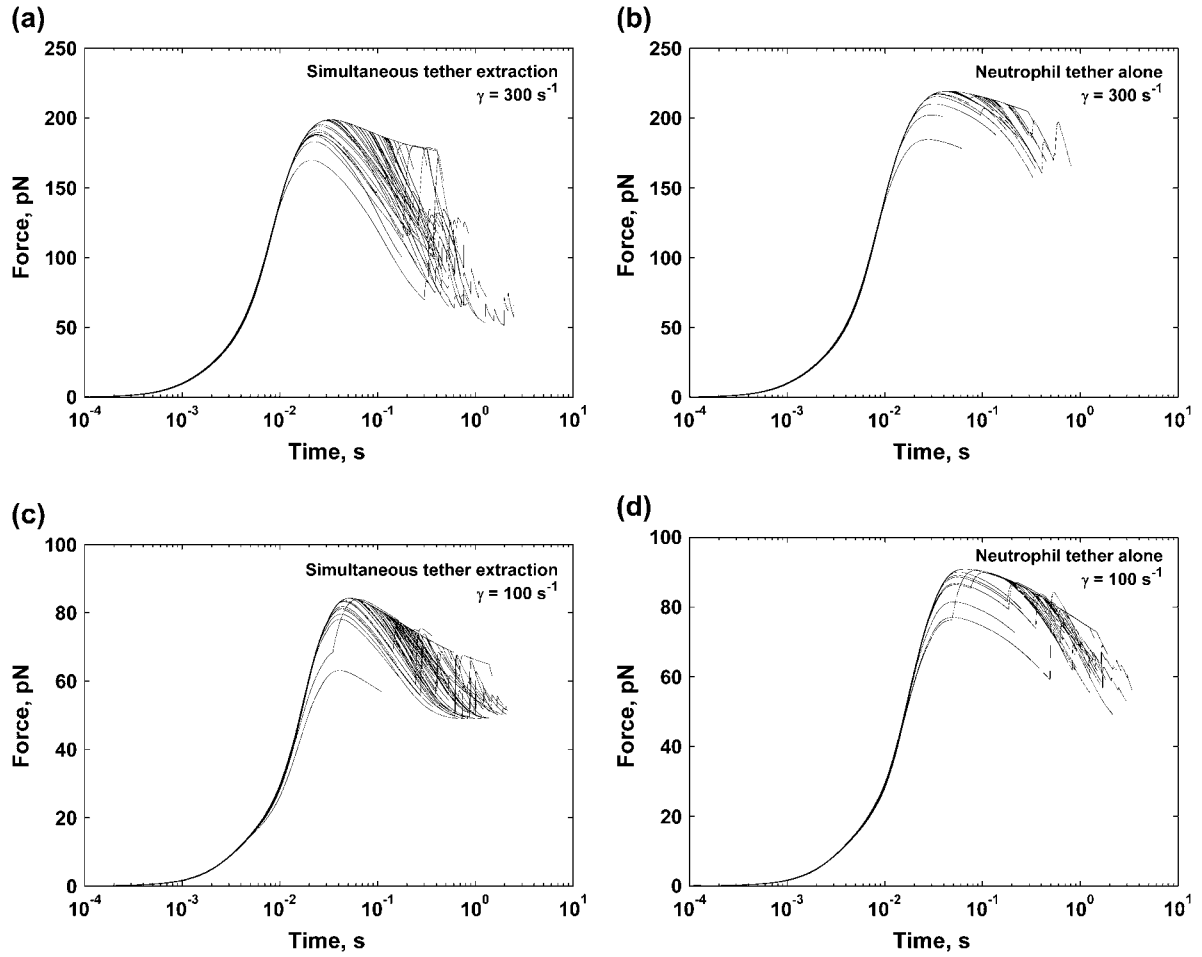

FIGURE 5 Effect of tether extraction on the force history of the first adhesive bond of all the simulated adherent cells at shear rates of 100 and 300 s^{-1} (each curve represents one cell). (*a* and *c*) Simultaneous tether extraction from the neutrophil and endothelial cell. (*b* and d) Tether extraction from the neutrophil alone. The density of P-selectin is 12 molecules/μm^2.

similar trends in force history and tether length were observed (16).

Fig. 6, *a–d*, shows the growth of the first initial tether of all the adherent cells (transient or stable rolling) at shear rates of 100 and 300 s^{-1} for two cases: simultaneous tether extraction (Fig. 6, *a* and *c*) and tether extraction from the neutrophil alone (Fig. 6, *b* and *d*), respectively. Compared to tether extraction from the neutrophil alone, the total tether length in the case of simultaneous tether extraction at 300 s^{-1} is about three times longer at similar time points. It is clear that endothelial cells contributed much more to the composite tether length because of their lower effective viscosity during tether extraction. The initial tethers in the case of simultaneous tether extraction at 300 s^{-1} also lasted longer (Figs. 6, *a* and *b*, and Fig. 7) and showed more variability in the family of curves shown in Fig. 6 *a*. On the contrary, the tether growth curves of all the adherent cells in the case of tether extraction from the neutrophil alone at 300 s^{-1} almost collapsed into one (Fig. 6 *b*). Tethers were extracted much more slowly at 100 s^{-1} than at 300 s^{-1}, so the advantage of simultaneous tether extraction over tether extraction from the neutrophil alone disappeared at 100 s^{-1}, because short tethers provide little stabilizing effect to the neutrophil rolling.

Fig. 7 shows the initial bond lifetime for the two tether extraction cases considered in Fig. 6. Because of the low ligand density on the substrate (12 molecules/μm^2), often only one bond was formed on the initially attached microvillus. Thus, the initial tether duration can also be interpreted as the initial bond lifetime most of the time. When the shear rate was increased, the initial bond lifetime would decrease. However, the decrease in the initial bond lifetime of the simultaneous tether case occurs much more slowly than in the case of the neutrophil tether alone. Consequently, when simultaneous tethers were extracted, the initial bond lifetime was consistently longer at shear rates of 200–500 s^{-1}. However, the two groups of data at the shear rate of 100 s^{-1} do not have any statistically significant difference. This is probably because, at this shear rate, the maximum force on the initial attached microvillus is only ~80 pN, which is just

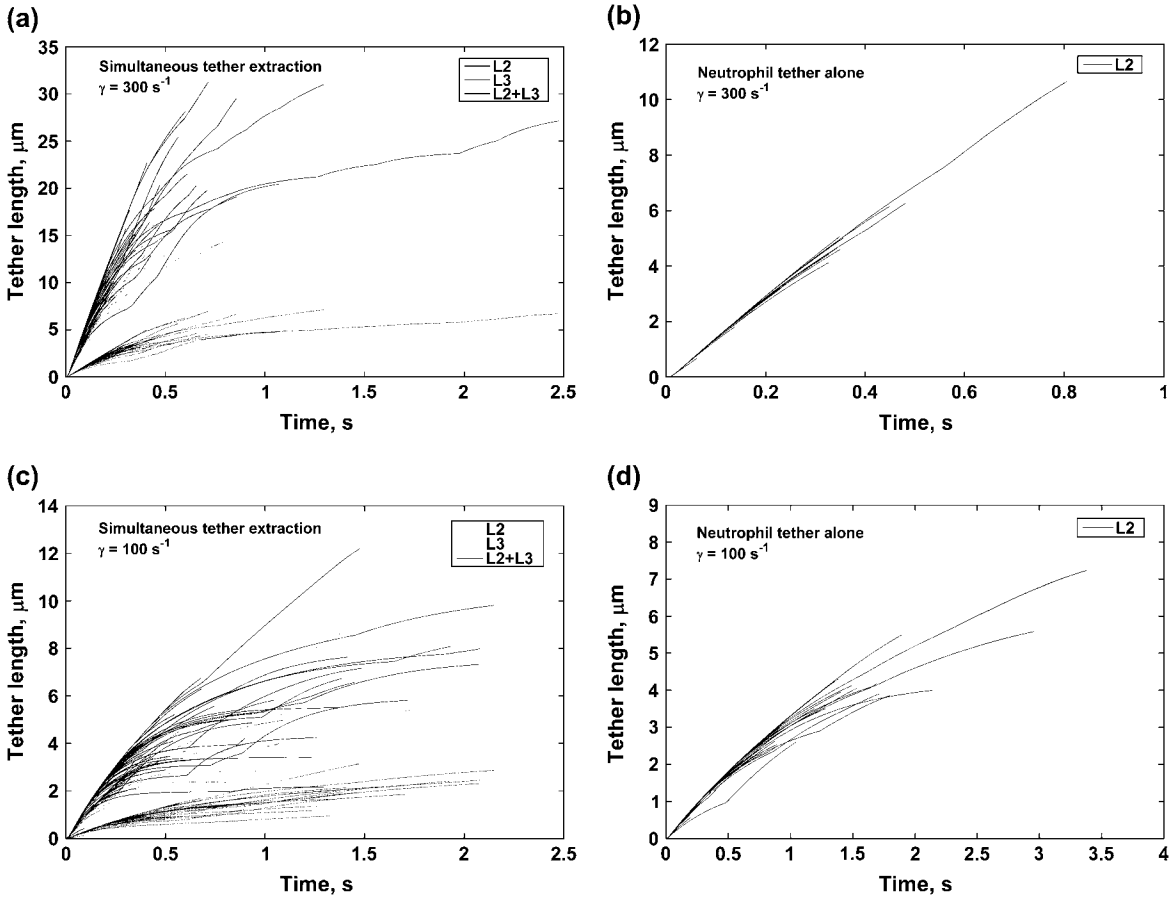

FIGURE 6 The growth of the initial tether at shear rates of 100 and 300 s^{-1} for simultaneous tether extraction from the neutrophil and endothelial cell (*a* and *c*) and for tether extraction from the neutrophil alone (*b* and *d*). L_2 and L_3 are defined in Fig. 1.

slightly larger than the threshold force for tether extraction from the neutrophil or endothelial cell, ~50 pN. Therefore, at the shear rate of 100 s^{-1}, the difference in the tether lengths between simultaneous tether extraction and tether extraction from the neutrophil alone was small (Fig. 6, *c* and *d*), so the membrane tethers did not have time to contribute significantly to the stabilization of the rolling.

Compared with tether extraction from the neutrophil alone, simultaneous tether extraction greatly decreases the force on the adhesive bond while increasing the probability of more microvillus adhesion to the substrate. Consequently, neutrophils that experience simultaneous tether extraction have a higher stable rolling percentage and more tethers while rolling, as shown in Fig. 8, *a* and *b*. In the case of simultaneous tether extraction, the average number of tethers on stable rolling cells did not increase as expected at a shear rate of 500 s^{-1}, but rather decreased. At higher shear rates, the adhesive tethers can be broken faster and the stabilizing effect of the tethers is diminished. Therefore, our results showed clearly that, compared to neutrophils rolling with tether extraction from the neutrophil alone, the cells with simultaneous tethers have a longer bond lifetime, a higher stable rolling percentage, and more tethers during rolling. As a result, they rolled much more stably.

Neutrophils rolling with simultaneous tether extraction are more shear-resistant

In all the simulations described above, the shear rates were constant during each simulation. To investigate how rolling neutrophils resist shear effectively, we conducted another simulation where cells were first allowed to adhere at a small shear rate, after which the shear rate was increased linearly every 1 s. The percentage of the adherent cells remaining after each increase was calculated and plotted at each shear rate for two different concentrations of P-selectin, as shown in Fig. 9 *a*. Clearly, the cells with simultaneous tether extraction were more shear-resistant, especially at high shear rates. The average number of tethers during rolling was also calculated at each shear rate, as shown in Fig. 9 *b*. Again, the difference between the cells with simultaneous tethers and those with neutrophil tether alone is obvious at high shear rates, but no significant difference is observed at low shear

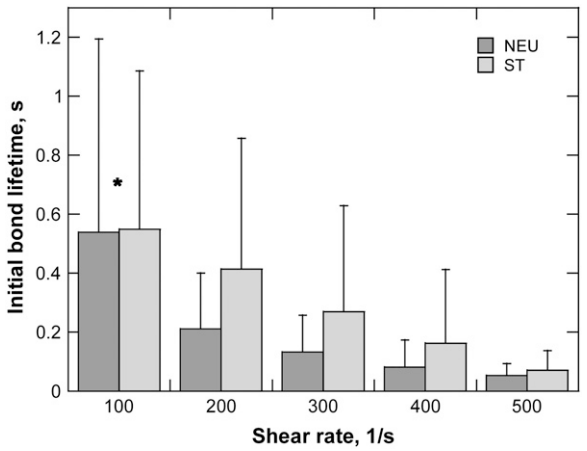

FIGURE 7 The initial bond lifetime for simultaneous tether extraction (*ST*) and neutrophil tether alone (*NEU*) at shear rates of 100–500 s^{-1}. There is no significant difference statistically between the two groups of data marked by the asterisk.

rate, which indicates that the contribution of simultaneous tethers is more crucial at high shear rates.

DISCUSSION

In this article, we have developed a two-dimensional model to simulate neutrophil rolling on the endothelium. In contrast to the rolling models developed earlier (15,31–39), we have focused solely on the contribution of membrane tether extraction, especially simultaneous tether extraction, to the stabilization of rolling. Our simulation reproduced some phenomena that have been observed in flow-chamber experiments (9,13). First, in contrast to the rolling of fixed neutrophils, tether extraction from the neutrophil allows the neutrophil to roll stably, with a small variance in rolling velocity. Second, compared with tether extraction from the neutrophil alone, simultaneous tether extraction from the neutrophil and endothelial cell greatly increases the initial bond lifetime, allows more transient tethers to make the transition into stable rolling, and enables rolling cells to be more shear-resistant. However, it has been shown experimentally that neutrophils roll with nearly constant velocity over a wide range of shear stress. Although simultaneous tether extraction from the neutrophil and endothelial cell is included in our model, we could not reproduce an asymptotic response of the rolling velocity at high shear stresses, which has been observed in flow-chamber experiments (40,41). Furthermore, the results shown in Fig. 8 indicate that tether stabilization alone cannot balance the effect of hydrodynamic forces at higher shear stress. Therefore, this implies that cellular deformation is critical for the decrease in rolling velocity at high shear stresses. Neutrophils that initially roll unstably at high shear stresses may develop stable rolling as they deform against the endothelial wall.

Our model, which constrains the cell motion in the *xz* plane (Fig. 1), focuses on simultaneous tether extraction

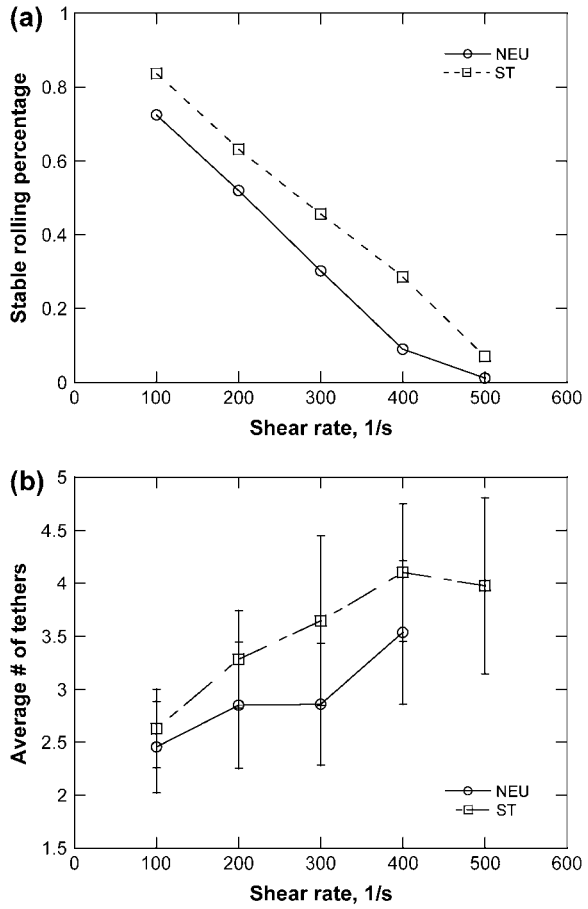

FIGURE 8 The percentage of stable rolling cells (*a*) and the average number of tethers on stable rolling cells (*b*) at shear rates of 100–500 s^{-1} for two cases: simultaneous tether extraction (*ST*) and neutrophil tether alone (*NEU*). At the shear rate of 500 s^{-1}, we excluded the data from the case of the neutrophil tether alone, because only one cell among 100 reached the stable rolling stage.

during the early stages of rolling, where neutrophils do not deform appreciably from their spherical shape. As a result, the model may not be applied to the latter stages of rolling. If the initial adhesive bond is formed out of the *xz* plane in a flow chamber or in vivo and the cell is pulled in a direction unparallel with the *xz* plane, the blood or fluid flow will quickly realign the cell with the microvillus into another plane, which is equivalent to the original *xz* plane shifted to a new position. This is because of the low Reynolds number nature of this problem and the large flexibility of the microvillus. For multiple tethers, an ideal model would be a three-dimensional one that allows the neutrophil to move in the *y* direction. However, the two-dimensional constraint should not significantly affect how forces are shared among multiple tethers, as long as these tethers are independent of each other mechanically. For example, if the second tether is also extracted out of the *xz* plane, the cell will again quickly reach the new equilibrium position. The tethers will be pulled in directions almost parallel with the *x* direction because most

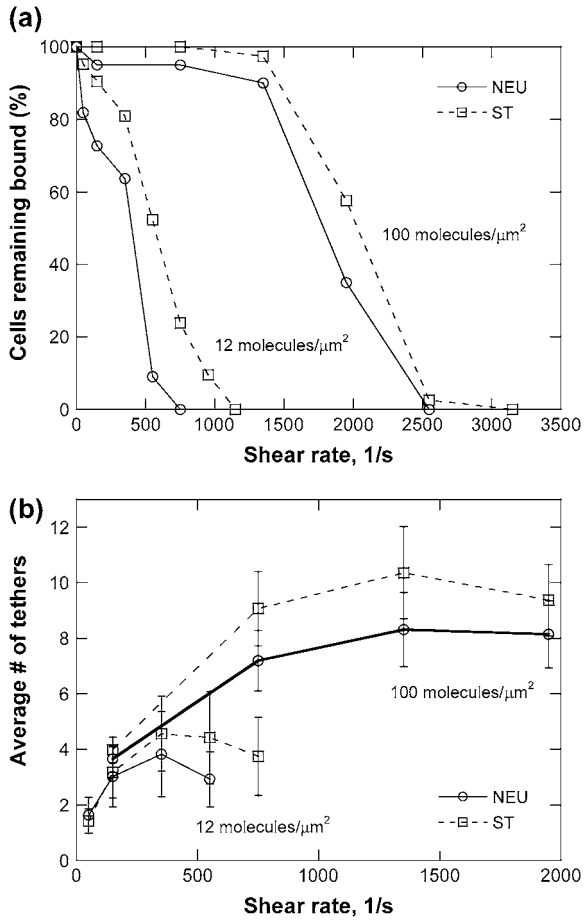

FIGURE 9 The percentage of cells remaining at increasing shear rates (*a*) and the average number of tethers (*b*) for tether extraction from the neutrophil alone (*NEU*) and simultaneous tether extraction from the neutrophil and endothelial cell (*ST*). Two concentrations of P-selectin on the substrate were simulated: 12 and 100 molecules/μm^2. At the smaller P-selectin concentration, cells were first allowed to adhere at the shear rate of 50 s^{-1}. The shear rate was then increased to 150, 350, 550, and 750 s^{-1} successively after every second. For ST and NEU, 31 and 32 adherent cells, respectively, were studied. At the larger P-selectin concentration, cells were first allowed to adhere at the shear rate of 150 s^{-1}. The shear rate was then increased by 600 s^{-1} every 1 s. Forty adherent cells were studied for each tether type, ST and NEU. At each shear rate, the percentage of remaining adherent cells was counted and the average number of tethers during rolling was calculated.

adhesive bonds will be formed at locations not too far from the contact point ($\sim$1 μm, according to our estimate) between the spherical cell and substrate. Consequently, the contributions of these tether forces to the overall mechanical equilibrium will be similar to the case where tethers are extracted exactly in the *xz* plane. At the latter stages of rolling, where more neutrophil deformation occurs, more tethers may form further away from the *xz* plane, but how these tethers affect neutrophil rolling can only be better simulated with a three-dimensional model.

In the simulation of normal neutrophils, P-selectin/PSGL-1 bonds are assumed to be 100 nm long. Molecular extension is ignored because the microvillus is likely to be much more elastic than P-selectin and PSGL-1, as well as their bond. Microvilli could be bent freely in the simulation because they could be easily pressed down to the neutrophil surface with a force of tens of piconewtons, which indicates a very small bending stiffness (11). Once microvilli are stretched, binding between the free receptors on the microvillus tip and the free ligands on the endothelial cell is unlikely, because the pulling force increases rapidly and pulls the receptors away from the ligands quickly. Evans et al. recently demonstrated that P-selectin/PSGL-1 bonds dissociate via two structure-dependent pathways (42). When P-selectin/PSGL-1 bonds were probed by linearly increasing forces over a range of force loading rates from 300 to 30,000 pN/s, the Bell model was used to describe the dissociation along a single pathway impeded by a sharp free-energy barrier. From the force history of the initial tether (Fig. 5, *a* and *b*), we can see clearly that the force first increased from zero to $\sim$200 pN within 0.1 s. At higher wall shear rates, the force increased to a larger value in even less time. Therefore, the initial force loading rate lies in the range of the single dissociation pathway, and it is reasonable to expect that the Bell model can be used to describe the dissociation of P-selectin/PSGL-1 bonds. However, as shown in Fig. 5, the force history on the adhesive bond is complex. The Bell model may not be appropriate for a small force loading rate, which can result from the slowly decreasing force on the bonds due to tether extraction. In addition, several experimental studies have shown the biphasic behavior of the P-selectin/PSGL-1 bond dissociation (43–45), which should be incorporated into the model in the future.

The minimum gap between the neutrophil and endothelial cell surface is assumed to be 30 nm, which is based on a correlation between the pressure (Δp) and the apparent gap (ε) obtained with the MAT (46). When a neutrophil is pushed toward another cell or substrate, the contact stress is defined to be the ratio of the contact force to the contact area. Based on the previously obtained correlation between Δp and ε, the range of the apparent gap between the cell and substrate may be from 0.01 to $\sim$0.1 μm at a contact force ranging from 250 to 150 pN (47). In our model, although the contact force depends on the wall shear rate and the properties of the attached microvilli, such as the contact angle (ϕ) and the total tether length (L_m), it probably lies in the above range. In fact, the force and torque imposed on the neutrophil are insensitive to the variation of the gap value between 0 and 0.5 μm, with $R = 4.25$ μm for the neutrophil, so we chose a constant value (30 nm) as the minimum gap between the neutrophil and endothelial cell (28,48).

In contrast to the effective viscosity of tether extraction from the neutrophil (1.8 pN·s/μm), the effective viscosity of tether extraction from the endothelial cell is only $\sim$0.5 pN·s/μm. As a result, tether extraction from the endothelium proceeds much more rapidly. At the same pulling force, the tether extraction velocity from the endothelial cell is around

three times that from the neutrophil. For simultaneous tether extraction, the tether from the endothelial cell contributes about three-fourths of the total composite tether length. However, tether extraction from the endothelial cell in the rolling process has not been fully investigated experimentally in vivo or in vitro. Most of the previous studies have been focused on the contribution of tether extraction from the neutrophil. Although our simulation of neutrophil rolling on the endothelium showed clearly how simultaneous tether extraction helps stabilize the neutrophil rolling, further experimental studies are still necessary to prove its existence and its stabilizing effect during the inflammatory response of the neutrophil.

CONCLUSIONS

Tether extraction from neutrophils during their rolling was first predicted from a micropipette aspiration study, and the stabilizing effect of tether extraction on neutrophil rolling was then confirmed in flow-chamber experiments. Tether extraction from endothelial cells was recently predicted to further stabilize neutrophil rolling on the endothelium. Therefore, in this study, we developed a numerical model to investigate the contribution of membrane tethers to neutrophil rolling stabilization. We first simulated fixed and normal neutrophils rolling on ligand-coated substrates to investigate the effect of tether extraction from the neutrophil alone. The simulation results showed that normal neutrophils with tether extraction rolled stably, with a small variance in rolling velocity, whereas fixed neutrophils rolled unstably, with a large variance in rolling velocity. This is consistent with what was observed in the flow-chamber experiments. We then simulated normal neutrophils rolling either on ligand-coated substrates or on endothelial cells to study the effect of simultaneous tether extraction from both the neutrophil and endothelial cell. Our results showed that simultaneous tether extraction was accompanied by longer initial bond lifetimes, higher stable rolling percentages, and stronger resistance to high shear rates. Therefore, we conclude that tether extraction from both neutrophils and endothelial cells contributes to the stabilization of neutrophil rolling.

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